Alanine-scanning Mutations in the "Primer Grip" of p66 HIV-1 Reverse Transcriptase Result in Selective Loss of RNA Priming Activity*

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Alanine-scanning mutants of the primer grip region of human immunodeficiency virus type 1 reverse transcriptase were tested for their ability to extend RNA and DNA versions of the polypurine tract primer, and an oligonucleotide representing the 18-nucleotide sequence at the 3' end of tRNA^{Lys3}. A majority of the mutant enzymes were either completely or severely deficient in RNA priming activity, but, with only one exception, were able to efficiently extend DNA versions of the same primers. The mutant enzymes were able to bind to RNA primers, indicating that the defect in RNA priming was not simply a loss of binding activity. Mutations at positions 229, 233, and 235 dramatically reduced the amount of specific RNase H cleavage at the 3' terminus of the polypurine tract, which is required for primer removal. An alanine substitution at position 232 led to loss of cleavage specificity, although total activity was close to the wild-type level. Taken together, these results demonstrate for the first time that there are residues in human immunodeficiency virus type 1 reverse transcriptase which are specifically involved in protein-nucleic acid interactions with RNA primers.

During the replicative cycle of human immunodeficiency virus (HIV)[1] and other retroviruses, reverse transcriptase (RT) catalyzes the conversion of single-stranded genomic RNA into linear double-stranded DNA, which is ultimately integrated into the host chromosome (Ref. 1; for reviews, see Refs. 2–4). Reverse transcription begins with initiation of minus-strand DNA synthesis from a cellular tRNA primer (reviewed in Refs. 5 and 6) bound to the primer binding site (PBS) at the 5' end of the viral RNA (2). In this step, RT must recognize and extend an RNA primer annealed to an RNA template (7, 8). Initiation of plus-strand DNA synthesis requires recognition of a purine-rich viral RNA sequence known as the polypurine tract (PPT). After specific RNase H cleavage at its 3' end, the PPT serves as

the primer for extension on a minus-strand DNA template (for review, see Ref. 9). The specificity of this reaction is provided, in part, by the inability of RT to prime plus-strand synthesis with RNA fragments lacking the PPT sequence (10–13). Thus, to initiate synthesis of each DNA strand, RT specifically recognizes RNA primers, which have exacting sequence requirements and are extended on defined templates.

In the case of HIV, formation of a specific (minus-strand) initiation complex between tRNA^{Lys3}, genomic RNA, and RT is followed by transition to an elongation mode of synthesis (14–16). Elongation of minus-strand DNA involves extended interactions between the RNA template and tRNA primer (15, 17) and also requires binding of RT to the 3'-OH of the growing DNA chain. As RT traverses the genomic RNA, it occasionally dissociates from the primer-template. To continue synthesis, it must therefore bind to different DNA primers on what is essentially a continuously-changing RNA template. Similarly, as elongation of plus-strand DNA proceeds, RT interacts with various DNA primers on a continuously changing DNA template. Thus, during elongation reactions, RT must be able to recognize DNA primers without regard to nucleotide sequence or configuration of the template.

These considerations make it clear that at different steps in reverse transcription, RT is presented with primer-template combinations having different helical structure, geometry, and nucleic acid composition. In view of these variations, it is conceivable that RT may have evolved different mechanisms or structural features to selectively recognize RNA and DNA primers.

Analysis of the x-ray crystal structure of HIV-1 RT complexed with a short duplex DNA primer-template indicates that the residues which interact with nucleotides at the 3' end of the primer constitute the β12-β13 hairpin in the p66 palm known as the "primer grip" (Fig. 1; Refs. 18 and 19). Residues in the primer grip line one side of a hydrophobic pocket to which the non-nucleoside inhibitor nevirapine binds (Ref. 20; for review, see Ref. 21). Interestingly, there are no nevirapine-resistant mutants with changes at residues Phe-227, Trp-229, and Leu-234. This suggests that these residues may be important for maintaining the structural integrity of the primer grip. Additionally, it has been shown that mutation of residues Trp-229, Met-230, Gly-231, and Tyr-232 results in alterations of both polymerase and RNase H activities (22).

In earlier work on the determinants of HIV-1 plus-strand priming, we showed that nucleotides at the 3' end of the HIV-1 PPT primer are critical for initiation of plus-strand DNA synthesis (13). Replacing the four G nucleotides at the 3' end of the PPT with four C nucleotides, rendered the PPT completely inactive as a primer for plus-strand synthesis. In view of the

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[1] The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; PBS, primer binding site; PPT, polypurine tract; WT, wild-type; nt, nucleotide(s).

This paper is available on line at http://www.jbc.stanford.edu/jbc/

proximity of the primer grip to the 3' end of the primer (18, 19), these findings suggested that the primer grip may have a specific role in RT-catalyzed initiation of plus-strand DNA synthesis.

The present study is focused on identification of residues that might be involved in unique interactions with the PPT primer. Our approach was to test the effect of introducing alanine substitutions within the primer grip on plus-strand priming. Our results show that mutations in this region can profoundly affect the ability of HIV-1 RT to extend an RNA PPT primer, while having little or no effect on priming with a DNA version of the PPT. Interestingly, most of these same mutations dramatically affect the ability of RT to initiate minus-strand DNA synthesis with an RNA oligonucleotide containing the 3' 18 terminal nucleotides of the tRNA^{Lys3} primer (RNA PBS primer). With only one exception, these mutations do not have a significant effect on extension of a DNA PBS primer. Thus, it appears that residues in the primer grip region play a specific role in recognition and extension of RNA primers.

EXPERIMENTAL PROCEDURES

Materials—RNA oligonucleotides were purchased from Oligos Etc., Inc. (Wilsonville, OR). Other materials were used as described previously (13, 22).

Construction and Purification of Alanine-scanning Mutations of the HIV-1 RT Primer Grip—Alanine substitutions in residues Glu-224 to His-235 in HIV-1 RT were constructed using *BcgI* cassette mutagenesis as described previously (23). The p66 subunits of mutant RTs were expressed separately and reconstituted with wild-type (WT) p51 to form p66/p51 heterodimers; thus, the mutation in each RT was present solely in the p66 subunit (24, 25). Reconstituted heterodimers were purified by metal chelate (Ni^{2+}-nitrilotriacetic acid-Sepharose) chromatography followed by ion exchange over S-Sepharose (26). The L234A mutant RT failed to reconstitute into a heterodimer (22) and was not studied further. The final RT preparations were stored in a 50% glycerol-containing buffer at −20 °C (26).

Oligonucleotide Assays for Initiation of Plus-strand DNA Synthesis—The ability of each RT to initiate plus-strand DNA synthesis was tested in an assay using synthetic RNA and DNA oligonucleotides, as described previously (13). Four primer-template combinations (Table I) were used (see schematic representation at the bottom of Fig. 2). The templates were all 35-nt DNA oligonucleotides. The primers were as follows. (i) A 15-nt RNA PPT already containing the 3' end normally generated after specific cleavage by the RNase H activity of WT RT was used to test the ability of RT to extend the PPT without requiring prior cleavage. (ii) A 20-nt RNA PPT containing the PPT sequence and the five bases immediately downstream of the PPT was used to test the ability of RT to specifically cleave the PPT prior to extension. (iii) Downstream RNA, a non-priming sequence was used. This RNA oligonucleotide contains the 15-nt sequence immediately downstream of the PPT. It does not function as a primer with WT HIV-1 RT (13) and serves as a negative control. (iv) A 15-nt DNA version of the PPT was used to test for primer extension with a DNA primer. Each reaction contained 1 pmol of primer-template and 10 pmol of WT or mutant HIV-1 RT. The products were internally labeled by addition of [α-^{32}P]dATP during synthesis. The reactions (total volume, 15 μl) were carried out at 37 °C for 15 min and were terminated by addition of formamide STOP solution. Reaction products were then heated to 95 °C for 5 min prior to loading on an 8% sequencing gel and visualization by autoradiography.

Determination of Catalytic Rate Constants with RNA and DNA PPT Primers—Catalytic rate constants for primer extension with a 15-nt RNA or DNA PPT primer were determined for WT RT and mutants E224A, P225A, and L228A. The time course for RNA PPT primer extension was performed essentially as described under "Oligonucleotide Assays for Initiation of Plus-strand DNA Synthesis," except that the reaction volume was increased 3-fold to 45 μl and 5-μl aliquots were removed at 1, 2, 3, 4, 5, 6, 10, and 15 min. Each 5-μl aliquot was added to 2 μl of formamide STOP solution. Since primer extension with a DNA PPT was very rapid (<1 min to reach completion) under the conditions of enzyme excess used in our standard assay, it was necessary to dilute each enzyme by 1:50 (0.2 pmol); this results in a molar ratio of enzyme to primer-template of 1:5. Dilutions of RT greater than 1:50 were not used since low enzyme concentrations are associated with an increase

in pausing (data not shown). Reactions were carried out as described above with the RNA PPT primer. However, in reactions with WT RT and the DNA PPT primer, time points were also taken at 15, 30, and 45 s to provide a more accurate curve-fit.

Samples were analyzed on an 8% sequencing gel, as described under "Oligonucleotide Assays for Initiation of Plus-strand DNA Synthesis." The amount of radioactivity incorporated into the 20-nt DNA product for the RNA PPT or the 35-nt product for the DNA PPT was determined by phosphorimaging, using the Molecular Dynamics STORM system. Values were converted to picomoles incorporated by comparison of the counts at each time point to counts from a control reaction containing WT RT incubated with the DNA PPT primer for 15 min (under standard assay conditions). Under these conditions, maximal incorporation is achieved in <1 min. Since 1 pmol of template is used in each reaction and each template is extended by 20 nt, the total incorporation is 20 pmol of nucleotides. The values from three independent experiments were averaged and then plotted as picomoles of nucleotides incorporated *versus* time in min. The resulting data were then fit to a single exponential equation, using the general curve-fitting routines found in the Macintosh program KaleidaGraph.

Rate constants were determined as described by Beard and Wilson (27), based on a model for DNA polymerase I developed by Bryant *et al.* (28). Since the rate constants for the RNA PPT primer extension were determined under conditions of 10-fold enzyme excess and represent the relatively slow rate of initiation, the values are independent of enzyme concentration. In this case, the rates are expressed only as the derived rate constants from the exponential fit. However, initiation was not as slow for DNA PPT-primed extension and the reaction conditions had to be altered (see above). In this case, primer-template is in excess and the rate constants represent the apparent turnover rates ($k = v_i/[RT]$), where v_i is the initial rate determined from the curve-fit and [RT] is enzyme concentration in picomoles.

Band-shift Assays—Binding to primer-template duplexes was investigated with two types of bandshift assays, essentially as described by Guo *et al.* (29). (i) The first type was binding only. One pmol (~2 × 10^4 total counts per min) of 5' end labeled "band-shift DNA template" (Table I) was annealed to 10 pmol of the 15-nt RNA or DNA PPT primers. Binding conditions for the band-shift assay were the same as those used in the oligonucleotide assay, except that dNTPs and [α-^{32}P]dATP were omitted. After incubation at 37 °C for 15 min, glycerol was added to a final concentration of 20% (v/v) and 10-μl portions of each reaction were loaded onto a 6% native polyacrylamide gel. Electrophoresis was carried out at room temperature for 60 min at a constant voltage of 200 V in a buffer containing 25 mM Tris-HCl and 162 mM glycine, pH 8.0. (ii) The second type was binding plus extension. Binding and extension were tested in the same band-shift assay by incubating an unlabeled DNA PPT primer-template (Table I) under the conditions used in the oligonucleotide assay, except that [α-^{32}P]dATP was the only dNTP added. The first base downstream from the PPT is a thymidine (see Table I). Thus, in this assay only primer-template that has been extended by one base will be labeled (29).

"trans" Assay for Specific Cleavage of a PPT-containing Substrate—Since most of the mutant RTs were unable to extend the RNA PPT primer, an assay was developed to test for the ability to catalyze specific cleavage at the 3' terminus of the PPT in *trans*. The 15-nt PPT primer was annealed to the PPT template (Table I) and extended using T4 DNA polymerase and internal labeling with [α-^{32}P]dATP, as described previously (13). Where specified, 10 pmol of WT or mutant RT was then added in *trans* and incubated with 1 pmol of substrate in a final volume of 15 μl for 15 min at 37 °C. The products were analyzed by polyacrylamide gel electrophoresis, as described above under "Oligonucleotide Assays for Initiation of Plus-strand DNA Synthesis."

Minus-strand Strong-stop DNA Synthesis—The ability of each mutant to initiate minus-strand strong-stop DNA synthesis was tested using a PBS-containing RNA template (30) annealed to either an RNA or DNA primer which is complementary to the PBS. The conditions used were the same as those previously described (22), except that the enzyme concentration was increased to achieve a 10:1 molar ratio of RT to primer-template. Synthesis was allowed to proceed for 60 min at 37 °C. Production of full-length strong-stop DNA on this RNA template results in a 192-nt DNA product (see Fig. 7).

RESULTS

Mutations in the Primer Grip Affecting the Ability of RT to Extend an RNA PPT Primer, but Not a DNA PPT Primer—In an earlier study, we developed a simple oligonucleotide assay to test for the ability of HIV-1 RT to initiate plus-strand DNA

synthesis from the 3' PPT (13). We used this assay to screen alanine-scanning mutants of the primer grip (residues 224–235; Fig. 1; Ref. 18) with four different primer-template combinations (see Table I and the bottom of Fig. 2): (i) a 15-nt RNA PPT primer, to measure specific extension; (ii) a 20-nt RNA PPT primer, to measure specific cleavage and subsequent extension; (iii) a non-PPT containing RNA oligonucleotide, which serves as a negative control; and (iv) a 15-nt DNA version of the PPT. The results are shown in Fig. 2 as *lanes 1–4*, respectively, for each enzyme.

WT HIV-1 RT readily extended both the 15- and 20-nt RNA PPT oligonucleotides to produce a specific 20-nt plus-strand DNA product (Fig. 2, *WT, lanes 1* and *2* and the schematic shown below). In the case of the 20-nt primer, cleavage of the five additional downstream bases occurs prior to extension (13). As previously observed (10–13), HIV-1 RT was unable to extend an RNA primer consisting of a non-PPT containing downstream sequence (Fig. 2, *WT, lane 3*). A DNA version of the PPT was efficiently extended, forming a 35-nt product consisting solely of DNA (Fig. 2, *WT, lane 4*).
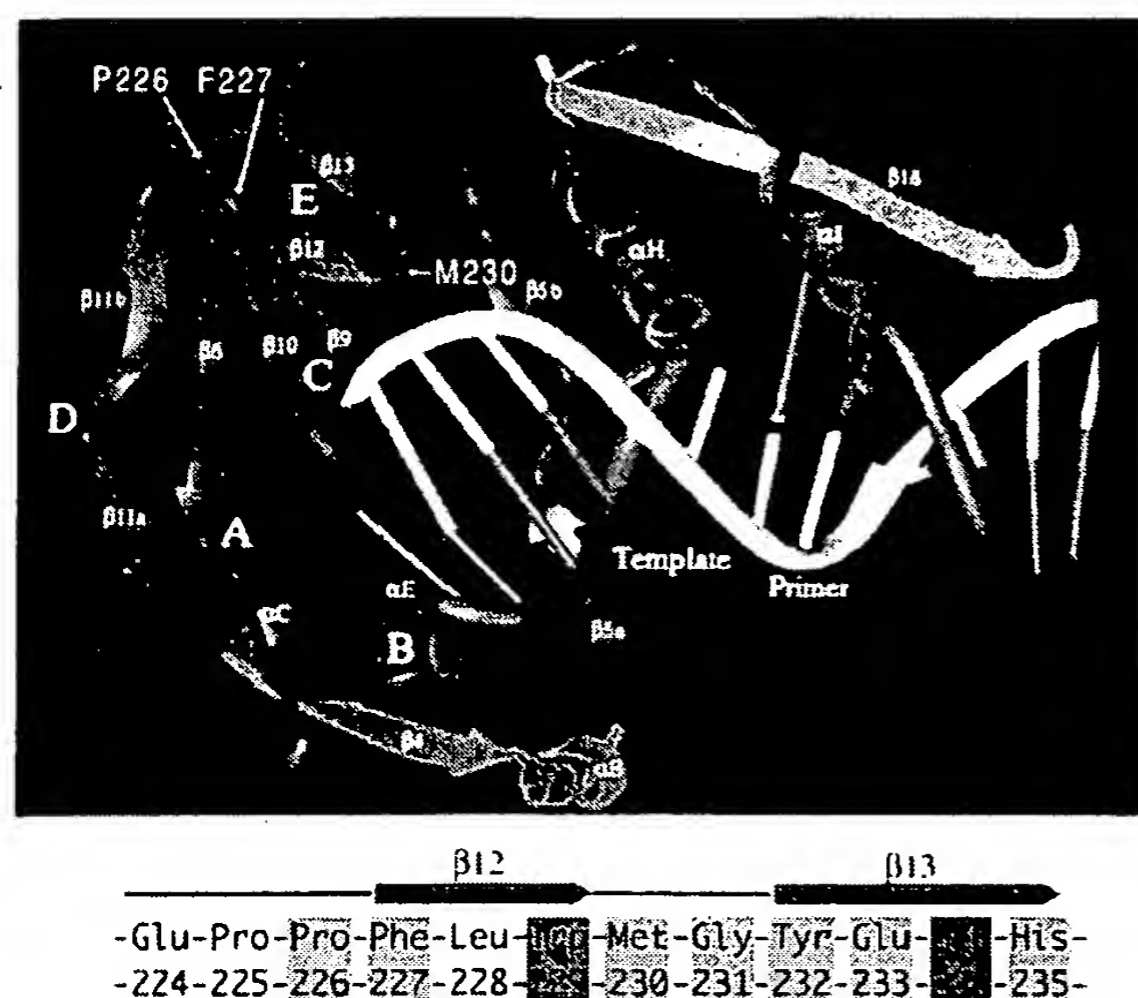

FIG. 1. **Structural features of p66 HIV-1 RT near the 3' end of the primer in a DNA-DNA primer-template (adapted from Fig. 3B in Ref. 18 with permission).** The primer grip is located in the β12-β13 hairpin (18). Residues 226, 227, and 230 are marked by *arrows* for reference. Underneath the ribbon diagram is a schematic representation of the residues mutated to alanine in this study (residues 224–235). Mutation of residues marked with a *yellow box* leads to specific defects in plus-strand initiation with an RNA PPT primer. Mutation of residue 229, marked with a *blue box*, results in a major defect in polymerase activity with RNA and DNA primers. The alanine substitution mutation at residue 234, marked with a *red box*, was not analyzed (see "Experimental Procedures"). Mutation of residues that are unmarked results in essentially WT levels of plus-strand initiation activity under the conditions of the standard assay.

When we tested the primer grip mutants using these same assays, we found that 8 of the 11 mutants (*i.e.* P226A, F227A, W229A, M230A, G231A, Y232A, E233A, and H235A) lost the ability to extend an RNA PPT primer (Fig. 2, *lanes 1* and *2* for each enzyme). In the case of P226A, a very small amount (<5% of WT) of plus-strand DNA product was detectable. All mutants, with the exception of W229A, extended a DNA version of the PPT primer as efficiently as WT under standard assay conditions (Fig. 2, *lane 4* for each enzyme). The W229A mutant was previously reported to have defective polymerase activity with a primer-template containing non-viral sequences (23). Thus, it appears that mutations at positions 226, 227, 229, 230–233, and 235 have a specific effect on recognition of the RNA PPT primer. As is the case for the WT enzyme, none of the mutants was able to extend the non-PPT containing RNA oligonucleotide (*lane 3* in each set; Ref. 13).

Changes in reaction conditions such as an increase in incubation time (up to 60 min) or concentration of the enzymes (up to a 60:1 ratio of RT to primer-template) had no effect on the amount of plus-strand product made by mutants that were inactive in the standard assay (data not shown). This suggests that the specific defect in ability of these RTs to recognize the RNA PPT is not kinetic in nature, but instead represents a loss in ability to add a base to the primer terminus.

Mutants E224A, P225A, and L228A behaved essentially like WT HIV-1 RT in our standard end point assay (Fig. 2, *lanes 1–4* of *E224, P225,* and *L228*). However, kinetic analysis of priming activities with the 15-nt RNA PPT primer revealed that the catalytic rate constants determined for mutants P225A and L228A were approximately 4- and 6.5-fold lower, respectively, than the value for WT RT, $k = 0.52$ min^{-1}; the rate constant for mutant E224A was very similar to that for the WT (Fig. 3A). Results from this analysis also revealed that, although the overall time for completion of the reaction is relatively long (on the order of min), no intermediate-size products were observed (data not shown). This suggests that once the RNA primer is extended, subsequent elongation and cleavage must occur very rapidly.

Similar analysis of the time course of primer extension with the 15-nt DNA PPT primer indicated that under our standard conditions where RT is in excess, reactions with mutants E224A, P225A, L228A, and WT RT proceeded too rapidly for determination of rate constants (data not shown). Thus, for these determinations, we modified the assay conditions and reduced the amount of each enzyme by 50-fold; this results in a 1:5 ratio of enzyme to primer-template. The catalytic rates for each of the mutant enzymes with the DNA PPT primer were similar ($k \cong 1.30$ min^{-1}) and were approximately 4-fold lower than that determined for WT RT (Fig. 3B).

Binding of the Primer Grip Mutants to Both RNA and DNA PPT Primer-Templates—To test the possibility that the primer grip mutants which could not prime plus-strand DNA synthesis with the RNA PPT are unable to bind to unextended primer-

TABLE I
Oligonucleotides used in this study

| Designation	Sequence	Type
15-nt PPT	5'-AAAAGAAAAGGGGGG	RNA
20-nt PPT	5'-AAAAGAAAAGGGGGGACUGG	RNA
DNA PPT	5'-AAAAGAAAAGGGGGG	DNA
PPT template	3'-TTTTCTTTTCCCCCCTGACCTTCCCGATTAAGTGA	DNA
Band shift template	3'-TTTTCTTTTCCCCCCTGA	DNA
Downstream RNA	5'-ACUGGAAGGGCUAAU	RNA
Downstream template	3'-TGACCTTCCCGATTAAGTGAGGGTTGCTTCTGTTC	DNA
RNA PBS primer	5'-GUCCCUGUUCGGGCGCCA	RNA
DNA PBS primer	5'-GTCCCTGTTCGGGCGCCA	DNA
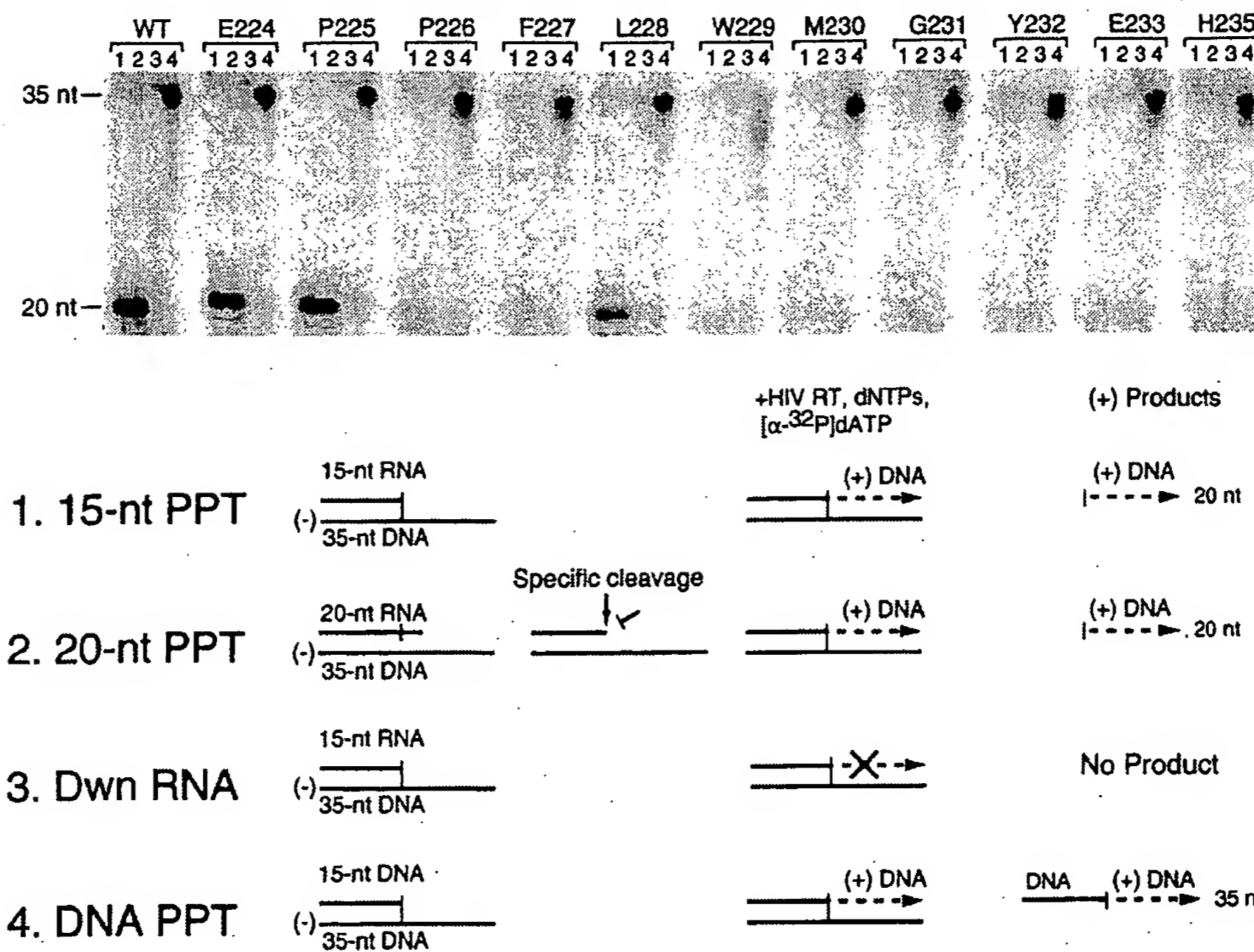
FIG. 2. **Oligonucleotide assay of plus-strand DNA synthesis.** Each primer grip mutant was tested with four different primer-template combinations (see Table I), as described under "Experimental Procedures": (i) 15-nt RNA PPT, *lane 1*; (ii) 20-nt RNA PPT, *lane 2*; (iii) downstream (*Dwn*) RNA, *lane 3*; and (iv) 15-nt DNA PPT, *lane 4*. The schematic diagram underneath the figure summarizes the activity tested by each primer-template combination and the expected DNA products.

template, we performed band-shift assays (29) using the RNA and DNA PPTs as primers (Fig. 4). We tested each mutant for the ability to form a stable complex under the same conditions used in the standard assay for plus-strand DNA synthesis (*i.e.* at a 10:1 ratio of RT to primer-template).

All of the primer grip mutants were able to form at least small amounts of a stable complex with the RNA PPT primer-template, although the fraction of shifted complex obtained with the mutant RTs was not as great as that seen with WT RT (Fig. 4A). In the case of mutant Y232A, the complex was barely detectable (Fig. 4A).

Interestingly, variation in binding of the different mutant RTs to the RNA PPT primer-template did not strictly correlate with their ability to extend this primer. For example, mutants E224A and P225A could extend the RNA PPT (Fig. 2), but the extent of binding seen with these RTs was similar to that of F227A (Fig. 4A), which could not extend the RNA PPT (Fig. 2). The reduction in binding efficiency of the mutants to the DNA PPT primer-template was similar to that found with the hybrid containing the RNA PPT (Fig. 4B). In addition, when the ratios of enzyme to primer-template were increased above 10:1 for these mutants, most of the RNA primer-template could ultimately be shifted to a stable complex (data not shown). Thus, it appears that the defect in the ability to extend the RNA PPT is not simply a loss of ability to bind to the primer-template, but a loss in the ability to extend the bound primer.

Some mutants (*i.e.* P226A, M230A, G231A, Y232A, and H235A; Fig. 4B) showed relatively poor binding to the DNA PPT primer-template but were able to efficiently extend the DNA PPT primer (see Fig. 2). To investigate a possible correlation between the amount of stable complex formed in the band-shift assay with the ability to prime from the DNA PPT, we performed the band-shift assay under conditions in which binding and extension were tested in the same experiment (Fig. 5). The experimental conditions were the same as those used in the DNA PPT band-shift experiment (Fig. 4B), except that the

primer-template was unlabeled and [α-^{32}P]dATP was included to allow labeling by incorporation of dA at the +1 position. In this case, the primer-template is labeled only if the enzyme is able to bind to an extent necessary for incorporation of a single, labeled base (29).

More pronounced differences between the primer grip mutants were observed with this assay. While all of the label incorporated by WT RT was present in bound primer-template, most or all of the label incorporated by the mutants was in unbound *i.e.* "free" primer-template (Fig. 5). For example, mutants M230A, G231A, and H235A incorporated little or no label into bound primer-template. This indicates that these mutants were able to extend the primer by one base without forming a stable complex in the band-shift assay. Mutant W229A was unable to incorporate significant amounts of label into either free or bound primer-template, most likely reflecting the low processivity of this enzyme, also seen in other assays (23). Interestingly, the mutants (E224A, P225A, and L228A) that produced the most labeled, shifted complex, (although still less than WT RT), were also the only mutants that could extend the RNA PPT (Fig. 2, Table II).

Mutations in the Primer Grip Can Affect Specific Cleavage at the PPT—In addition to the investigation of plus-strand priming activity, it was of interest to test the ability of each mutant to remove the primer by specific RNase H cleavage at the 3' terminus of the PPT. Since most of the primer grip mutants are unable to extend an RNA PPT primer (Fig. 2), we used a two-step procedure for the cleavage assay. The 15-nt RNA PPT primer was first extended by T4 DNA polymerase to produce a 35-nt RNA-DNA chimeric product; then, either WT or mutant RT was added in *trans*. Removal of the primer by the RNase H activity of the RT being tested should generate a 20-nt DNA. A schematic diagram of the assay is presented at the bottom of Fig. 6.

WT HIV-1 RT efficiently cleaved the pre-extended primer to produce the expected 20-nt plus-strand DNA product (Fig. 6).

A   RNA PPT Primer
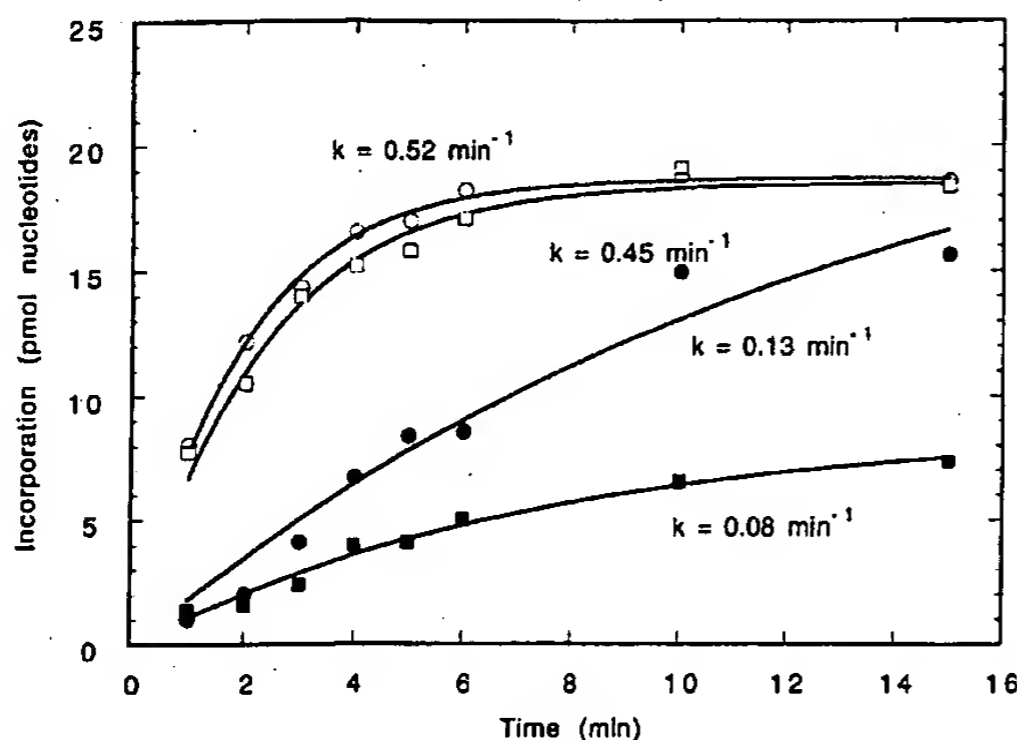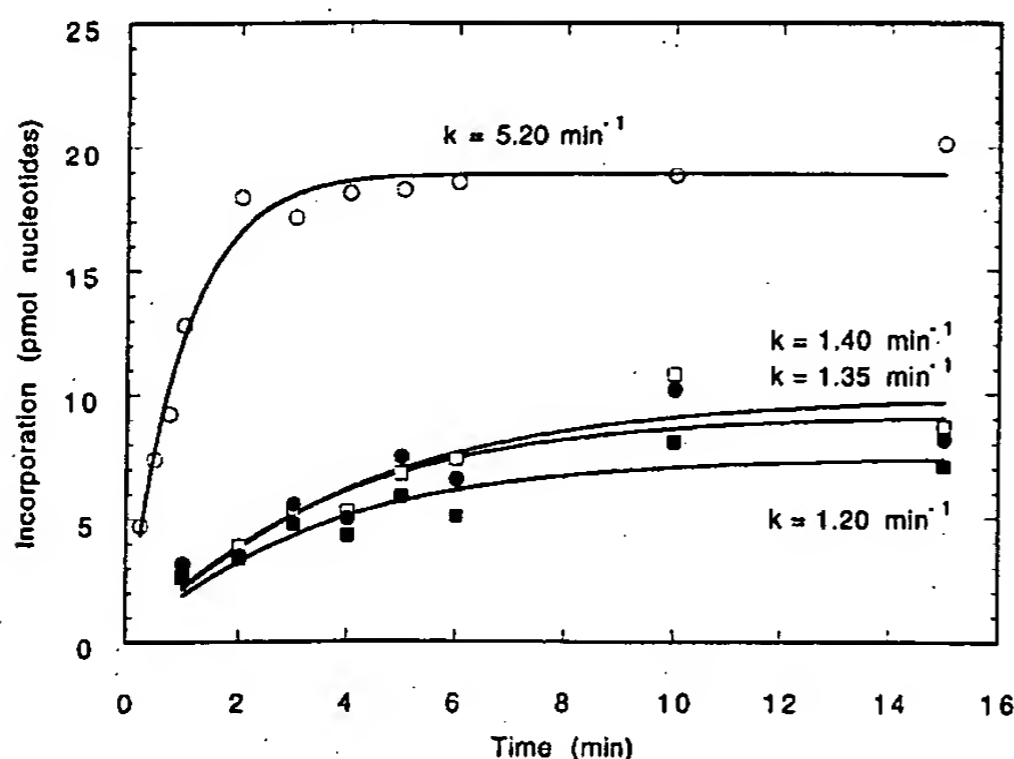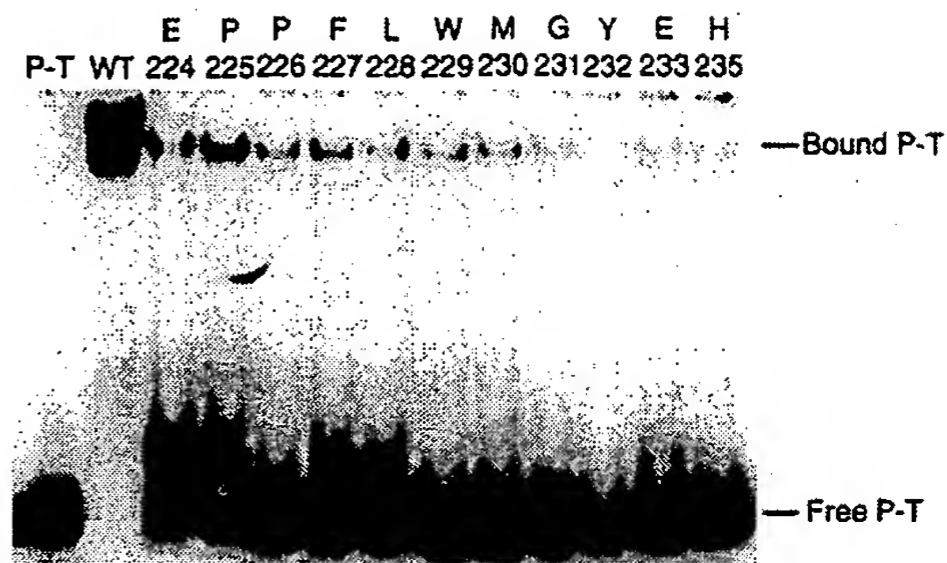

B   DNA PPT Primer

FIG. 3. **Kinetic analysis of plus-strand priming activities of WT RT and mutants E224A, P225A, and L228A.** Reactions were carried out with the 15-nt RNA or DNA PPT primers. Aliquots were removed at the indicated times, and samples were run in 8% sequencing gels. Catalytic rate constants were determined for each enzyme as described under "Experimental Procedures." The calculated values are shown on the figure above or below the appropriate curve. Each point represents the average of three independent measurements. *A*, RNA PPT primer; *B*, DNA PPT primer. *Open circles*, WT RT; *open squares*, E224A; *closed circles*, P225A; and *closed squares*, L228A. Note that the enzyme concentration used in *A* is 10 pmol, while that used in *B* is 0.2 pmol.

The activity of mutant RTs E224A and P225A was equivalent to that of WT RT. Mutants P226A, F227A, L228A, M230A, G231A, and Y232A all retained significant activity, but were not as efficient as WT RT. Mutant W229A exhibited less than half the activity of WT RT, while E233A and H235A had dramatically reduced cleavage activity (~20–25% of WT RT activity). In one case (Y232A), total RNase H activity was 85% of WT, but cleavage was not precise. Additional cleavages within the PPT generated products of 21 and 22 nt in addition to the specific 20-nt DNA product (Fig. 6, *Y232*). Table II summarizes densitometric quantitation of the 20-nt plus-strand DNA product produced by each mutant. The observation that each of the mutant RTs can catalyze removal of the primer, albeit with some variation in efficiency or precision of cleavage, indicates that all of the RTs were able to bind, at least to some extent, to the extended primer-template.

Activity of the Primer Grip Mutants with an RNA or DNA PBS Primer—To determine whether the inability of primer grip mutants to extend the RNA PPT represents a specific defect in RNA PPT recognition or a more general effect on RNA
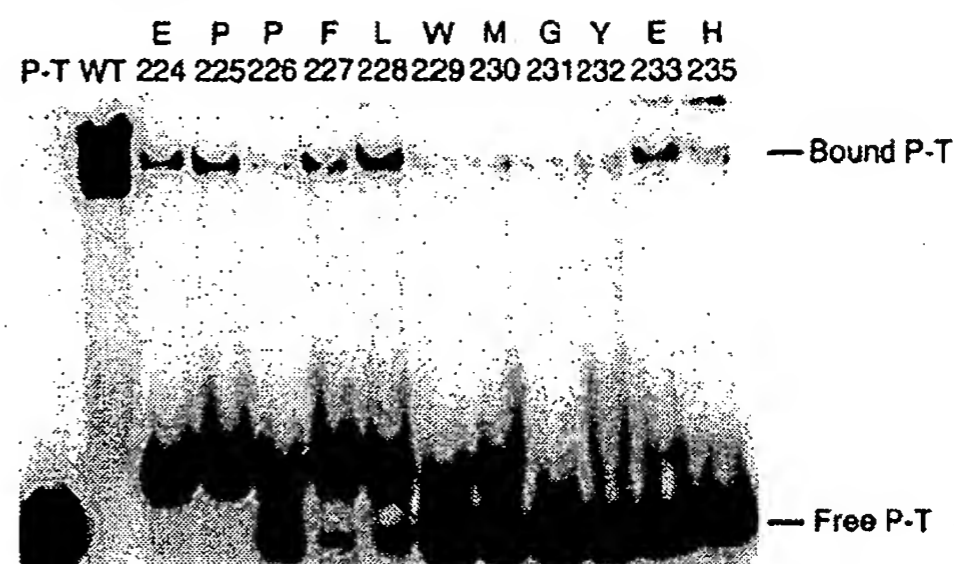
A   RNA PPT Primer

B   DNA PPT Primer

FIG. 4. **Band-shift assay showing binding of the primer grip mutants to RNA and DNA versions of the PPT primer.** *A*, 15-nt RNA PPT primer. *B*, 15-nt DNA PPT primer. The *lanes* labeled *P-T* are free primer-template with no RT added. In each case, the minus-strand DNA template was labeled with ^{32}P at its 5' end. Each enzyme was tested using the conditions described for plus-strand synthesis, except that the DNA template was shortened to 18 nt (Table I) and dNTPs were omitted.

primer recognition, we tested the ability of the mutant RTs to extend RNA and DNA versions of an 18-nt primer complementary to the PBS and having the sequence at the 3' end of tRNA[Lys3] (Fig. 7). In this case, the ability of the mutants to recognize either an RNA primer on an RNA template (RNA-RNA hybrid) or a DNA primer on an RNA template (DNA-RNA hybrid) is being measured.

In agreement with our observations with the RNA PPT-containing substrate (Fig. 2, Table II), only mutants E224A, P225A, and L228A could extend the RNA PBS primer to approximately the same extent as WT HIV-1 RT (Fig. 7*A*, compare *lanes 2, 3*, and *6* with *lane 1*). In this assay, however, mutants P226A, F227A, and E233A were able to produce significant amounts of full-length minus-strand DNA, although not as efficiently as WT RT (compare *lanes 4, 5*, and *11* with *lane 1*). Mutant M230A was able to produce a small amount of full-length minus-strand DNA and some shorter products (*lane 8*), while mutant G231A made a small amount of short minus-strand products and virtually no full-length DNA (*lane 9*). Mutants W229A, Y232A, and H235A were completely deficient in minus-strand DNA synthesis (*lanes 7, 10*, and *12*).

The activities of mutant and wild-type RTs were also tested with a DNA version of the PBS primer (Fig. 7*B*). In agreement with the results seen with the DNA version of the PPT primer (Fig. 2, Table II), extension of the DNA PBS primer resulted in significant synthesis of full-length DNA in all cases, except with mutant W229A (*lane 7*).

Taken together, the results presented in this paper demonstrate that substitution of alanine in most of the residues in the primer grip region of HIV-1 RT selectively affects the ability to
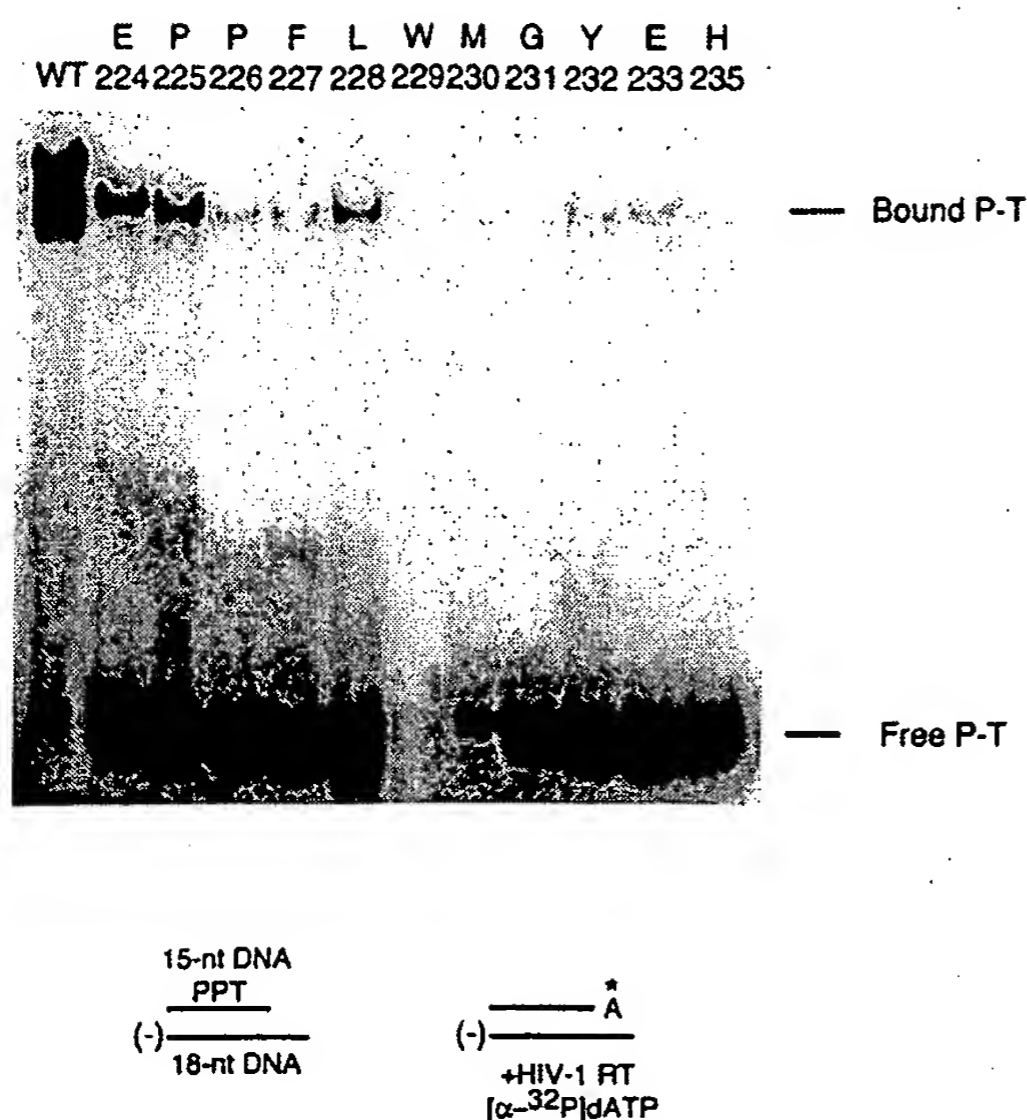
FIG. 5. **Band-shift assay showing binding of primer grip mutants to a 15-nt DNA PPT primer-template under conditions which allow extension of the primer by a single nucleotide.** The conditions of this assay are the same as those described in the legend to Fig. 4, except that $[\alpha^{-32}P]dATP$ was added to the binding mixture to allow a +1 extension of the DNA PPT primer. A schematic diagram of the assay is presented at the bottom of the figure. In this assay, only primer-template that is extended by RT will be labeled (29). Note that many of the enzymes can incorporate label into unbound *i.e.* free primer-template.

TABLE II
Summary of the activities of primer grip mutants

Enzyme	Extension of				PPT removal[a]
	RNA PPT	DNA PPT	RNA PBS	DNA PBS	
WT	+ + + +[b]	+ + + +	+ + + +	+ + + +	100
E224A	+ + + +	+ + + +	+ + + +	+ + + +	107
P225A	+ + + +	+ + + +	+ + + +	+ + + +	102
P226A	+/−	+ + + +	+ +	+ + + +	80
F227A	−	+ + + +	+ + +	+ + + +	75
L228A	+ + +	+ + + +	+ + + +	+ + + +	67
W229A	−	+	−	+	39
M230A	−	+ + + +	+	+ + + +	73
G231A	−	+ + + +	+/−	+ + + +	67
Y232A	−	+ + + +	−	+ + + +	85[c]
E233A	−	+ + + +	+ + +	+ + + +	24
H235A	−	+ + + +	−	+ + + +	19

[a] The percentage of cleavage relative to WT (100%) was determined by densitometric analysis of the 20-nt DNA product generated after removal of the 15-nt RNA PPT primer in the *"trans"* assay (see "Experimental Procedures").

[b] −, 0%; +/−, <25%; +, 25%; + +, 50%; + + +, 75%; + + + +, 100%.

[c] Y232A was unable to catalyze precise cleavage at the PPT.

catalyze primer extension with an RNA primer. In contrast, with the exception of the W229A substitution, the mutations have little or no effect on polymerase activity with a DNA primer.

DISCUSSION

Analysis of the three-dimensional structure of heterodimeric HIV-1 RT complexed with a short duplex DNA primer-template indicates that nucleotides at the 3' end of the primer interact with residues 227–235 in the $\beta12$-$\beta13$ hairpin in the p66 palm subdomain, a region known as the primer grip (Fig. 1; Refs. 18 and 19). In the present report, we investigated the effect of individual alanine substitutions in residues of the primer grip on the ability of RT to extend RNA PPT and PBS primers and
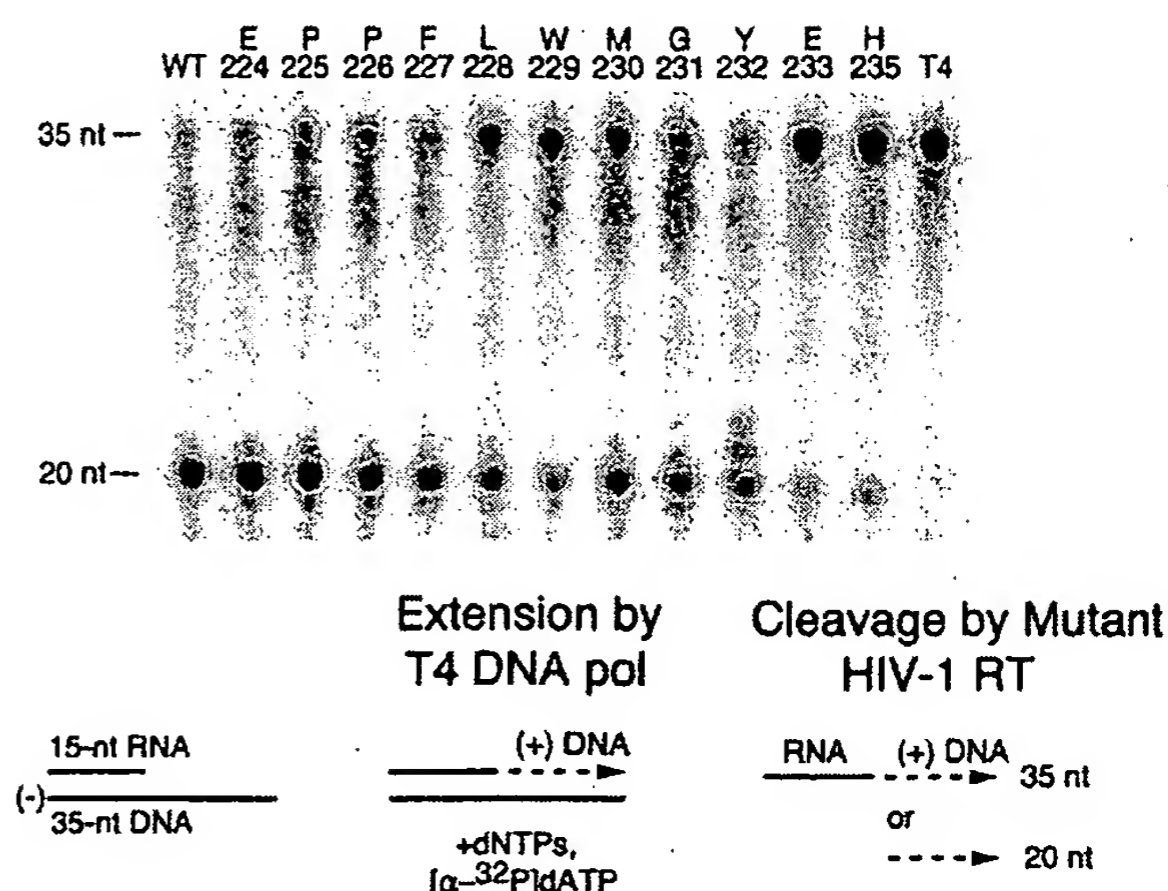

FIG. 6. **Assay of RNA PPT primer removal from an extended primer-template (*trans* assay).** The 15-nt RNA PPT primer-template was first extended by T4 DNA polymerase (which lacks RNase H activity). To determine the effect of the alanine substitutions on RNase H activity, each primer grip mutant was then added in *trans*, as described under "Experimental Procedures." The lane labeled T4 shows the DNA product synthesized following primer extension by T4 DNA polymerase, without subsequent addition of RT in *trans*. A schematic diagram of the assay is presented at the bottom of the figure.
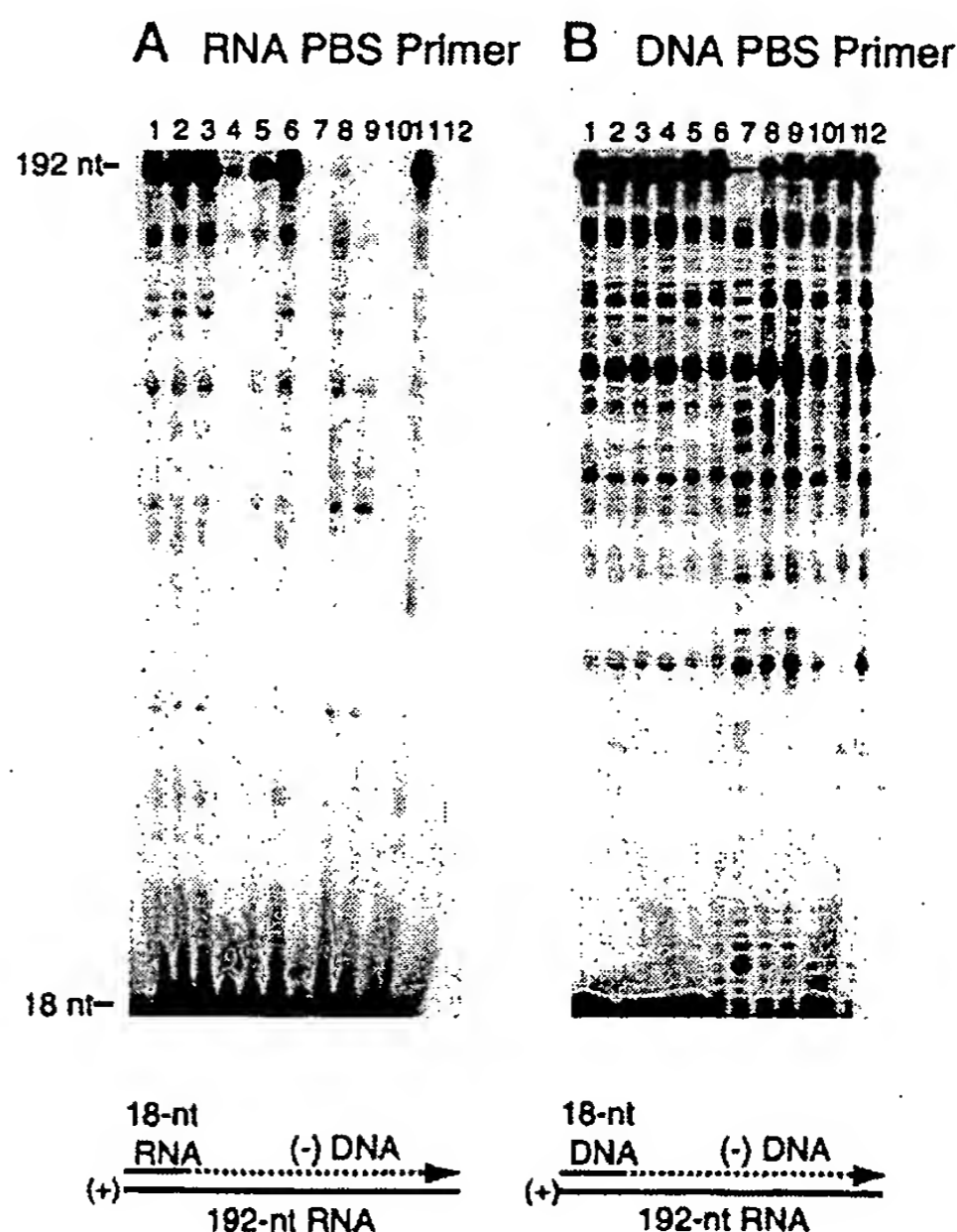

FIG. 7. **Ability of primer grip mutants to extend RNA or DNA versions of the PBS primer.** *A*, 18-nt RNA PBS primer; *B*, 18-nt DNA PBS primer. Reaction conditions for synthesis of minus-strand DNA are described under "Experimental Procedures." Schematic diagrams of each assay are shown at the bottom of each panel in the figure. *Lane 1*, WT; *lane 2*, E224A; *lane 3*, P225A; *lane 4*, P226A; *lane 5*, F227A; *lane 6*, L228A; *lane 7*, W229A; *lane 8*, M230A; *lane 9*, G231A; *lane 10*, Y232A; *lane 11*, E223A; and *lane 12*, H235A.

the corresponding DNA versions of these primers.

Almost all of the mutations in the primer grip residues have a drastic effect on RNA priming activity and with only one exception (W229A), have little or no effect on extension of DNA primers (Figs. 2, 3, and 7, Table II). Three mutants, E224A, P225A, and L228A, appeared to have wild-type activity with

Primer Grip Mutations Selectively Eliminate RNA Priming

the RNA PPT primer in our end point assay (Fig. 2). However, kinetic analysis revealed that mutants P225A and L228A have moderately lower catalytic rate constants than WT RT, while the rate constant determined for mutant E224A is essentially the same as that of WT (Fig. 3A). In two cases, F227A and E233A, the alanine substitution has a specific effect on recognition of the RNA PPT primer (Fig. 2), but not the RNA PBS primer (Fig. 7A).

In a parallel study (31), we have found that aromatic substitutions at positions 229 and 232, either singly or in combination, result in a phenotype similar to that of most of the alanine-scanning mutants. Thus, none of the aromatic mutants is able to initiate plus-strand synthesis with an RNA PPT primer and almost all are defective in initiation of minus-strand synthesis with tRNA^{Lys3} or an RNA PBS primer; in contrast, the mutants are able to extend DNA primers (31).

The present findings do not simply reflect an inability of the mutant RTs to bind to RNA primer-templates, since binding to the RNA and DNA PPT primer-templates was similar (Fig. 4). Mutants E224A, P225A, and L228A (the only mutants with the ability to extend the RNA PPT primer; Figs. 2 and 3) are also able to maintain a greater fraction of stable enzyme-primer-template complex after the addition of one base (Fig. 5). However, for many of the other mutants, the ability to incorporate a labeled dNTP is not correlated with formation of a stable complex with RT in the band-shift assay (Fig. 5). (This is also true of the Trp-229 and Tyr-232 aromatic substitution mutants (31).) Finally, although the W229A mutant is able to bind tRNA^{Lys3} as well as WT RT (23), it has no activity with the RNA PBS primer. These results imply that the primer grip mutations block a step subsequent to binding, possibly involving proper positioning of the RNA primer-template on the polymerase active site.

The results also suggest that interactions of the primer grip residues with RNA primers are qualitatively different from interactions with DNA primers. How can we account for this selective effect on RNA priming activity? Here, we consider the possibility that differences in the helical structures of hybrids containing an RNA or a DNA primer have an important influence on the way in which RT contacts the primer-template for subsequent primer extension. This idea is supported by results showing that the ability of tRNA^{Lys3} to function as a primer (to initiate minus-strand DNA synthesis) or as a template (to reconstitute the PBS during plus-strand DNA synthesis) is determined by the nature of the nucleic acid to which the tRNA is hybridized (8).

A DNA primer annealed to a DNA template (*e.g.* as is the case in plus-strand DNA elongation) will adopt a purely B-form helical structure (32, 33), while an RNA primer annealed to an RNA template (*e.g.* as is the case in minus-strand initiation) will adopt a strictly A-form structure (34). The situation for RNA-DNA hybrids, however, is somewhat more complex. Nuclear magnetic resonance studies have shown that the structure of RNA-DNA hybrids is neither strictly A- or B-form (35, 36). The sugars of the RNA strand adopt an A-form C3'-*endo* conformation, while the sugars in the DNA strand have an intermediate O4'-*endo* conformation (35, 36). An additional structural feature of a short purine-rich hybrid partially resembling the PPT is that its major groove is wider than that of other RNA-DNA hybrids and is close to the size of the major groove of B-type DNA duplexes; the purine-rich hybrid also contains a prominent bend in the double helix (36). These considerations indicate that the structure of the primer in an RNA-DNA hybrid will depend on whether the primer is the RNA or DNA strand and on its purine content.

Clearly, RT can recognize and extend different DNA primers on RNA and DNA templates, since this is precisely what occurs during elongation of minus- and plus-strand DNA, respectively. However, in the case of plus-strand initiation, which involves extension of an RNA primer on a DNA template, there are more stringent requirements and the PPT alone is selected from a potentially large number of possible RNA primers (10–13). One feature of the PPT that could contribute to its selection as a primer appears to be its unusual helical structure (13). We have shown that an RNA primer that has the same helical structure as the PPT, but a different primary sequence, can be efficiently extended by HIV-1 RT (13). Therefore, for extension to occur, it may be necessary for an RNA primer to adopt a particular helical structure, which facilitates contacts between the primer and residues in the primer grip. These contacts would presumably vary depending on whether an RNA or DNA primer was present in the hybrid, and it is possible that even minor changes in the primer grip are sufficient to disrupt RNA priming, while leaving DNA priming activity relatively unaffected.

Another question of interest in this study was whether alanine-scanning mutations in the primer grip have any effect on RNase H activity. Although the polymerase and RNase H active sites are localized to different domains of RT (37–42), mutations in the polymerase domain can also have an effect on RNase H catalytic activity (38, 42–44).

In earlier work, using a heteropolymeric substrate with a recessed DNA primer, it was shown that alanine substitutions at positions 224–229 had no effect on RNase H cleavage (23). More recent analysis (22) has revealed that mutants G231A and Y232A are deficient in catalyzing secondary cleavage of the template at the −8 position, *i.e.* at a position in the RNA corresponding to the eighth nucleotide from the 3' terminus of the primer (polymerase- or 3'-OH-independent cleavage; Refs. 43, 45, and 46). Indeed, mutant Y232A has the unusual phenotype of directly processing the substrate to a −8 product without going through the usual −17 intermediate (22). Using an RNase H assay that measures RNA 5' end-directed cleavage with a heteropolymeric substrate having a recessed RNA template (47, 48), it was found that in the presence of a heparin trap, mutants P226A, F227A, G231A, Y232A, E233A, and H235A are all inactive (49). Under these conditions, it could also be shown that mutants P226A and F227A are unable to bind to the substrate (49).

PPT primer removal by specific RNase H cleavage was analyzed in the present study, and the results indicated that certain primer grip mutations have an effect on this activity (Fig. 6, Table II). The most dramatic change in the level of specific cleavage was observed with mutants E233A and H235A, which had only 24% and 19% of the activity exhibited by WT RT, respectively. The W229A mutant also had reduced RNase H activity (39% of WT). Interestingly mutant Y232A had almost WT levels of activity; however, several products were produced, indicating that cleavage at the PPT was not precise. This observation supports the idea that a mutation at Tyr-232 loosens the grip on the substrate, thereby allowing multiple cleavages to occur. This result complements findings with other RNase H assays (22, 49), which also indicate that RNase H cleavage defects are associated with the Y232A mutation.

In summary, data presented in this study demonstrate that alanine substitutions at most positions of the primer grip in p66 HIV-1 RT reduce or eliminate the ability of RT to utilize RNA primers. With only one exception, alanine substitution has little or no effect on DNA priming activity. We propose that interactions of residues in the primer grip with RNA and DNA primers are qualitatively different and are determined, at least in part, by RT recognition of the helical structure of the hybrids

formed with these primers. A major implication of these findings is the importance of choosing several different biologically relevant primer-templates for assessing HIV-1 RT activity and screening potential RT inhibitors.

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